Bifunctional Fusion Protein with Thrombolytic and Anticoagulant Activities and Uses Thereof

Technical Field

This application relates to a fusion protein, which is composed of a thrombolytic protein, an anticoagulant protein, and a linker peptide. In particular, the fusion protein is composed of an anticoagulant protein and a protein molecule having plasminogen-activating activity, wherein said two proteins are linked together via a linker peptide, which can be recognized and cleaved by blood coagulation factors. The application also relates to the medical use of said fusion protein, and to the use of the linker peptide which can be recognized by blood coagulation factor in linking a thrombolytic protein and an anticoagulation protein.

Background of Invention

Cardiovascular diseases are the leading causes of deaths of human being. Presently, urokinase and tissue-type plasminogen activator are used in clinic as major thrombolytic drugs, while heparin and hirudin are used as major anticoagulant drugs. Although thrombolytic therapy is successful in decreasing the death rate of patients suffering from thrombus, re- thrombus will often occur due to the entering of small gore into blood circulation. Therefore, heparin or hirudin is now used as anticoagulant agents in combination with thrombolytic agents for thrombolytic therapy.

However, thrombolytic agents and anticoagulant agents usually cause systematic haemolysis and anticoagulation due to their poor specificity, thereby leading to systematic haemorrhage.

In particular, tissue-type plasminogen activator(t-PA), streptokinase(SK), urokinase(UK) and urokinase-like plasminogen activator(u-PA) can activate the plasminogen, which in turn lyses clots at the site of thrombus. However, the activated plasminogen will also cause bleeding in non-thrombus locus. Staphylokinase (SAK) is a new plasminogen activator of native origin, and has certain specificity to the thrombus.

Hirudin (HV), which is a small protein, is regarded as the new anticoagulant agent

due to its high affinity and selective inhibition to thrombin. However, hirudin is inclined to cause systematic haemorrhage in clinic. Moreover, there is no antagonist against hirudin hitherto.

Thus, to decrease side effects and increase therapeutic effects of thrombolytic drugs, it is important to increase their selectivity, targeting property and local concentration at thrombus clots, and to decrease drug concentration or activity at thrombus-free sites and the side effect of bleeding. The fusion protein in the prior art cannot reduce the side effect of bleeding.

Therefore, the development of anti-thrombus drug is focused on bifunctional medicament having both thrombolytic and anticoagulant activities. To combine thrombolytic activity with anticoagulant activity, the fusion of hirudin with SAK or SK have been studied. It showed that, if the N-terminal of hirudin was linked to C-terminal of SAK or SK, the hirudin will loss its anti-thrombin activity, while the plasminogen-activating activity of the thrombolytic protein is retained or partially retained; if the C-terminal of hirudin is linked to N-terminal of SAK, then due to the rapid degradation of N-terminal of SAK *in vivo*, the fusion protein will undergo degradation before functioning. Thus it is attracting in thrombolytic therapy to develop fusion protein having both thrombolytic and anticoagulant activities.

Purpose of Invention

The purpose of this invention is to provide a fusion protein having both thrombolytic and anticoagulant activities.

Disclosure of the Invention

This invention is based on the following recognition: a fusion protein can be constructed by linking a thrombolytic protein and an anticoagulant protein via a peptide containing blood coagulation factor-recognizable sequence. Thus constructed fusion protein provides several advantages as follows: Firstly, the fusion protein retains plasminogen-activating activity and thus the thrombolytic activity; Secondly, the N-terminal of the anticoagulant protein such as hirudin is linked to C-terminal of the thrombolytic protein, and thus the whole fusion protein doesn't show anticoagulant activity at thrombus-free sites and *in vitro*, thereby eliminating or reducing the side effect of bleeding caused by the anticoagulant protein such as

hirudin; Thirdly, the fusion protein has the ability of targeting to clots due to the high affinity of hirudin to thrombin at thrombus locus, and thus it increases drug concentration at thrombus locus and decreases the therapeutical amount of the drug; Fourthly, when the fusion protein circulates to the locus of thrombus, the blood coagulation factor, which is involved in thrombosis and is characteristic of the thrombus, rapidly cleaves the fusion protein at the designed recognition site, and relieves the free thrombolytic protein and anticoagulant protein, thereby functioning both thrombolytic and thrombolytic activities. The invention has been established in terms of the above advantages.

In one aspect, the invention relates to a fusion protein composed of a thrombolytic protein, anticoagulant protein and a linker peptide.

In another aspect, the invention relates to a method for preparation of a fusion protein comprising a thrombolytic protein and an anticoagulant protein, which method comprises linking a thrombolytic protein gene and an anticoagulant protein gene via a base sequence encoding IEGR or GPR to form a gene encoding said fusion protein, and expressing said gene encoding the fusion protein in E.coli, yeast or animal cell lines to produce the fusion protein.

In a further aspect, the invention relates to a pharmaceutical composition comprising said fusion protein and a pharmaceutical acceptable carrier or excipient.

In a further aspect, the invention relates to the use of linker peptide containing a sequence recognized by blood coagulation factor in the preparation of a fusion protein comprising a thrombolytic protein and an anticoagulant protein.

In a further aspect, the invention relates to the use of a sequence which can be recognized and cleaved by blood coagulation factor as a linker peptide of a thrombolytic protein and an anticoagulant protein.

In still further aspect, the invention relates to a method for treatment of diseases or conditions associated with thrombosis, which method comprises administrating a therapeutically effective amount of the fusion protein to the patient suffering from thrombosis.

Illustration of the drawings

The following drawings are presented to illustrate this invention, but they are not

intended to limit the invention.

Figure 1 is an illustration of the fusion protein.

Figure 2 shows the electrophoresis of the fusion protein gene, staphylokinase(SAK) gene and hirudin(HV2) gene.

As used herein, the term "thrombolytic protein" refers to the proteins having thrombolytic activity, for example staphylokinase(SAK), tissue-type plasminogen activator(t-PA), streptokinase(SK), urokinase(UK), urokinase-like plasminogen activator(u-PA), venom and mutants thereof which activate other hemolytic factors or have thrombolytic activity *per se*. Staphylokinase(SAK) or mutant thereof is preferred.

As used herein, the term "anticoagulant protein" refers to the proteins having anticoagulant activity, such as hirudin, antithrombin III, venom and mutants thereof. Hirudin or mutant thereof is preferred.

As used herein, the term "linker peptide recognized by blood coagulation factor" refers to the tetrapeptide of IEGR (IleGluGlyArg) , peptide containing IEGR sequence, tripeptide of GPR (GlyProArg) or peptide containing GPR sequence.

As used herein, the term "diseases or conditions associated with thrombosis" refers to any disease or condition caused by thrombus, such as cerebral thrombus, arterial thrombus, stroke and atherosclerosis.

As used herein, the term "patient" refers to mammals, particularly human being.

According to this invention, the fusion proteins of the invention is preferably a SFH fusion protein(SAK-GSIEGR-HV2) composed of staphylokinase and hirudin linked by GSIEGR, a fusion protein(tPA-PRIEGR-HV2) composed of tissue-type plasminogen activator(t-PA) and hirudin linked by PRIEGR, or a fusion protein(SAK-GSGPR-HV2) composed of staphylokinase and hirudin linked by GSGPR.

According to the invention, the fusion protein may be expressed in *E. coli, Pichia pastoris, Saccharomyces cerevisiae* or animal cells. Preferably, it is expressed in *E.coli* or yeast cells.

The following Examples are intended to illustrate the invention, but not mean to limit the invention.

Examples

Example 1 Preparation of fusion protein SFH (SAK-GSIEGR-HV2) and its bifunctional activities

EcoR I and BamH I restriction sites are added to the two ends of SAK gene, respectively. The SAK gene without the stop codon is introduced in the vector pBV220, resulting in pBVSAK. By PCR method, the BamH I restriction site and the sequence coding FXa recognition sequence GSIEGR are incorporated upstream of hirudin gene via a primer (5'-CG GGA TCC ATC GAA GGT CGT ATT ACT TAC ACT GAT TGT ACA GAA TCG-3'). The primer matched with downstream of the hirudin gene contains a Pst I restriction site. The hirudin gene with a FXa recognition sequence GSIEGR is digested by two enzymes of BamH I and Pst I, and the above vector pBVSAK is also digested by BamH I and Pst I. The digested hirudin fragment is inserted into the digested vector pBVSAK to form plasmid pBVSFH (see Figure 1). Said two gene fragments can also been linked by overlapping PCR method. The plasmid pBVSFH is transformed into *E.coli*, and induced to express at 42℃. The desired fusion protein (SFH) is obtained by ion exchange and gel filtration method in a purity of more than 96%. The SFH fusion protein comprises three domains, a SAK sequence, FXa recognition sequence GSIEGR and hirudin. The amino acid sequence of SFH fusion protein is as follows:

1 sssfdkgkyk kgddasyfep tgpylmvnvt gvdgkgnell sphyvefpik
61 pgttltkeki eyyvewalda taykefrvve ldpsakievt yydknkkkee
101 sfpitekg fvvpdlsehi knpgfnlitk viiekkgsie gritytdcte sgqdlclceg
161 snvcgkgnkc ilgsngeenq cvtgegtpkp qshndgdfee ipeeylq

The thrombolytic activity of the purified fusion protein was determined using chromogenic substrate S-2251. To test thrombolytic and anticoagulant activity of the fusion protein *in vivo*, mouse-tail thrombosis (RTT) was induced by kappa-carrageenin. The results show that the anticoagulant activity of the SFH fusion protein is significantly higher than that of SAK. In particular, after induction by kappa-carrageenin for 24 hrs, SAK is i.p. injected at a dose of 1.2mg/kg body weight

every eight hours, and the inhibition of the tail thrombus is 36.6%. However, when equimolar SFH is administrated at a dose of 1.8mg/kg body weight, the inhibition of the tail thrombus is 100%. After induction by kappa-carrageenin for 36 hrs, the inhibition of the tail thrombus reaches 18.2% and 90% respectively by SAK and SFH administrated as above. The detailed results are shown in Tables 1-3.

Table 1 Thrombolytic activity of fusion protein(SFH) and staphylokinase(SAK) determined by using chromogenic substrates (S - 2251)(Reaction time is 5min. n = 3, Δ OD_{405})

Samples	SAK	SFH
Δ OD_{405}	0.357±0.22	0.394±0.01

Note: 2nM fusion protein and 2nM staphylokinase were used for determination.

Table 2 Anticoagulant activity of fusion protein(SFH) activated by FXa

Samples	Anticoagulant activities
Fusion protein not cleaved by FXa	0
Fusion protein cleaved with 0.2U of FXa for 10min	2560ATU

Note:5.8μg of the fusion protein SFH and 0.2 U of FXa were co-incubated for 10min at 37°C. Clot method was used for determination of the anticoagulant activity.

Table 3 In vivo anticoagulation activities of the fusion protein SFH and staphylokinase (SAK) (n = 10)

kappa-carrageenin-inducing time (hr)	No. animals of each group	SAK	SFH
24	10	36.6%	100%

36	10	18.2%	90%

Note: SAK is i.p. administrated at a dose of 1.2mg/kg body weight every eight hours, and equimolar SFH is administrated at a dose of 1.8mg/kg. The animals used are Kunming mice(KM mice). The anticoagulant activity is expressed as the inhibition of mouse-tail thrombosis.

Table 1 shows that the fusion protein SFH exhibits the same level of thrombolytic activity as free staphylokinase. Table 2 shows that the intact fusion protein SFH does not exhibit anticoagulant activity, but shows entire anticoagulant activity once cleaved by the blood coagulant factor FXa. Table 3 demonstrates that SFH has the significant anticoagulant effect. Thus the fusion protein of the invention indeed has both the thrombolytic and anticoagulant activities.

Example 2 Preparation of fusion protein tPA-PRIEGR-HV2

Xho I and Avr II restriction sites are added to the upstream and downstream of tPA gene, respectively. The tPA gene without the stop codon is introduced in the vector pPIC9. The Avr II restriction site and the sequence coding FXa recognition sequence are incorporated upstream of hirudin gene via a primer using PCR method. The primer matched with downstream of the hirudin gene contains a Not I restriction site. The hirudin gene with a FXa recognition sequence is digested by two enzymes of Avr II and Not I, and the resulting fragment is linked into the above constructed vector pPIC9, wherein the introduced fragment is located downstream of tPA gene and forms together with tPA into the fusion gene PAFH. Thus constructed plasmid is designed as pPAFH. The plasmids pPAFH and pPIC9K are digested by BamH I and Sal I. The PAFH gene is then inserted into pPIC9K to form pPAFH-K gene. The plasmid pPAFH-K is linearized, and incorporated into yeast genome by electrotransformation. Methanol is used to induce the expression. The desired fusion protein comprises three domains, a tPA sequence, FXa recognition sequence and hirudin..

Example 3 Preparation of fusion protein STH (SAK-GSLGPR-HV2) and its bifunctional activities

EcoR I and BamH I restriction sites are added to the two ends of SAK gene, respectively. The SAK gene without the stop codon is introduced in the vector pBV220, resulting in pBVSAK. The BamH I restriction site and the sequence coding FXIIa recognition sequence GSLGPR are incorporated upstream of hirudin gene via a primer using PCR method. The primer matched with downstream of the hirudin gene contains a Pst I restriction site. The hirudin gene with a FXIIa recognition sequence GSLGPR is digested by two enzymes of BamH I and Pst I, and the above vector pBVSAK is also digested by BamH I and Pst I. The digested hirudin fragment is inserted into the digested vector pBVSAK to form plasmid pBVSTH. The sequence is confirmed by enzymatic digestion. Alternatively, said two gene fragments may be linked by overlapping PCR method. The plasmid pBVSTH is transformed into *E.coli*, and induced to express at 42℃. The desired fusion protein (STH) is obtained by ion exchange and gel filtration method in a purity of more than 96%. The STH fusion protein comprises three domains, a SAK sequence, FXIIa recognition sequence GSLGPR and hirudin.